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(54) Title: VIRULENCE ASSOCIATED PROTEINS IN *BORRELIA BURGDORFERI Bb*

(57) Abstract

The invention relates to a purified polypeptide having a molecular weight of about 38 kDa isolated from a virulent strain of *Borrelia burgdorferi*. The 38 kDa protein may be used for the detection of *B. burgdorferi* in human or animal tissues or body fluids, for immunization, and also for the generation of specific antibodies to *B. burgdorferi*.

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DESCRIPTION

VIRULENCE ASSOCIATED PROTEINS IN *BORRELIA BURGDORFERI (Bb)*

The present application is a continuation-in-part of co-pending U.S. Patent No. 5,245,844 which issued on September 21, 1993, the entire text of which disclosure is specifically incorporated herein without disclaimer. The United States Government may have certain rights in the present invention pursuant to Grant No. AI 24424 and Grant No. AI 29731 awarded by the National Institutes of Health.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to nucleic acid sequences encoding antigenic proteins associated with *Borrelia burgdorferi* (*Bb*), particularly polypeptides associated with virulence. The invention also relates to methods for producing *Bb* immunogenic polypeptides and corresponding antibodies. Other embodiments of the invention relate to methods for detecting Lyme disease and transformed cells comprising *Bb*-associated nucleic acids.

Description of Related Art

Borrelia burgdorferi (*Bb*) (Rahn and Malawista, 1991). Although recognized as a clinical entity within the last few

century. Cases of the disease have been reported in Europe, Asia and North America (Schmid, 1985). Despite a relatively low total incidence compared to other infectious diseases, Lyme disease represents a significant health problem because of its potentially severe cardiovascular, neurologic and arthritic complications, difficulty in diagnosis and treatment and high prevalence in some geographic regions.

There is increasing evidence that *Bb* is not a homogeneous group but has a variable genetic content, which may in turn affect its virulence, pattern of pathogenesis and immunogenicity. Its virulence factors, pathogenetic mechanisms and means of immune evasion are unknown. At the level of patient care, diagnosis of the disease is complicated by its varied clinical presentation and the lack of practical, standardized diagnostic tests of high sensitivity and specificity. Antimicrobial therapy is not always effective, particularly in the later stages of the disease.

Variation among *Bb* strains and the changes resulting from *in vitro* passage add to the problems of developing vaccines or immunodiagnostics from either the whole organism or specifically associated proteins. Using a PCR assay, it was found that one set of oligonucleotide primers was specific for North American *Bb* isolates, another for most European isolates and a third set recognized all *Bb* strains (Rosa *et al.*, 1989).

Serological assays for the diagnosis and detection

[illegible] and suffer from a low "signal to noise" ratio, i.e., a low degree of reactivity in positive samples, particularly early in the

results in high numbers of false negatives and the potential for false positives. Background reactivity in negative controls may be due in part to conserved antigens such as the 41K flagellin and the 60K "Common Antigen". These *Bb* proteins possess a high degree of sequence homology with similar proteins found in other bacteria. Therefore normal individuals will often express anti-flagellar and anti-60K antibodies. Unique, highly reactive *Bb* antigens for serological assays are therefore desirable but heretofore unavailable.

Diagnosis of Lyme disease remains a complex and uncertain endeavor, due to lack of any single diagnostic tool that is both sensitive and specific. Clinical manifestations and history are the most common bases for diagnosis. However, there is a pressing need for specific, sensitive, reproducible and readily available confirmatory tests. Direct detection offers proof of infection but is hampered by the extremely low levels of *Bb* that are typically present during infection, as well as the inaccessibility of sites that tend to be consistently positive (*e.g.*, heart and bladder). Culture, although sensitive, is cumbersome and requires 1-3 weeks to obtain a positive result. PCR appears to offer promise in terms of direct detection (Lebech *et al.*, 1991) and indeed Goodman *et al* (1991) have reported detection of *Bb* DNA in the urine of patients with active Lyme disease using a PCR method. However, it is unlikely that PCR assays will become commonly used in clinical laboratories because of the degree of skill required for

substantial number of humans exposed to *Bb* who develop inapparent or asymptomatic infections. This number has

There is clearly a need for means of preparing *Bb*-specific antigens, *e.g.*, for the development of diagnostic tests for Lyme disease. Adequate assays do not exist and should ideally meet several criteria, including (1) expression of an antigen by all pathogenic *Bb* strains, (2) elicitation of an immune response in all Lyme disease patients, (3) high immunogenicity with a detectable antibody response early in the infection stage, (4) antigens unique to *Bb* without cross reactivity to other antigens and, (5) distinction between individuals exposed to nonpathogenic as opposed to pathogenic forms of *Bb*.

SUMMARY OF THE INVENTION

The present invention addresses one or more of the foregoing or other problems associated with the preparation and use of *Bb*-specific antigens, particularly those antigens associated with virulence and which are useful for developing detection and diagnostic methods for Lyme disease. The invention involves the identification of such antigens, as well as the identification and isolation of *Bb* nucleic acid sequences that encode *Bb* antigens or antigenic polypeptides derived therefrom. These sequences are useful for preparing expression vectors for transforming host cells to produce recombinant antigenic polypeptides. It is further proposed that these antigens will be useful as vaccines or immunodiagnostic agents for *B. burgdorferi* associated diseases such as Lyme disease in particular.

[illegible] acid sequences associated with virulent *Bb*. These sequences are important for their ability to selectively hybridize with complementary

hybridization may be desired, depending on the application envisioned and the selectivity of the probe toward the target sequence. Where a high degree of selectivity is desired, one may employ relatively stringent conditions to form the hybrids, such as relatively low salt and/or high temperature conditions. Under these conditions, little mismatch between the probe and template or target strand is tolerated. Less stringent conditions might be employed where, for example, one desires to prepare mutants or to detect mutants when significant divergence exists.

In clinical diagnostic embodiments, nucleic acid segments of the present invention may be used in combination with an appropriate means, such as a label, to determine hybridization with DNA of a pathogenic organism. Typical methods of detection might utilize, for example, radioactive species, enzyme-active or other marker ligands such as avidin/biotin, which are detectable directly or indirectly. In preferred diagnostic embodiments, one will likely desire to employ an enzyme tag such as alkaline phosphatase or peroxidase rather than radioactive or other reagents that may have undesirable environmental effects. Enzyme tags, for example, often utilize colorimetric indicator substrates that are readily detectable spectrophotometrically, many in the visible wavelength range. Luminescent substrates could also be used for increased sensitivity.

Hybridizable DNA segments may include any of a

desired, longer segments including 25, 30 or 40 base pairs, depending on the particular applications desired.

are generally preferable for blot hybridizations. It should be pointed out, however, a certain amount of variation or base substitution would be expected, *e.g.*, as may be found in mutants or strain variants, but which do not significantly affect hybridization characteristics. Such variations, including base modifications occurring naturally or otherwise, are intended to be included within the scope of the present invention.

In embodiments relating to antigen production, DNA segments are disclosed that encode an antigenic polypeptide derived from the amino acid sequence of an antigen of *Bb*. Particularly preferred for such an application is the 30 kDa *Bb* antigen. However, it is proposed that various other *Bb*-specific antigens have been identified and may be obtained employing the procedures disclosed herein, including, *e.g.*, the 35 kDa, 24 kDa, and 20 kDa antigens. Until now, antigenic proteins appearing to be uniquely associated with virulence, such as the 30 kDa species, have not been isolated, purified or characterized. Through the present invention, recombinant means to obtain *e.g.*, the 30 kDa protein and its epitopes in useful amounts have been provided. It is particularly noteworthy that the invention provides for the identification and selection of antigens such as the 30 kDa antigen that are associated with low passage, virulent *Bb* strains so that a selective detection method for virulent strains of *Bb* is now possible.

will be found to vary from isolate to isolate. Moreover, it is quite clear that changes may be made in the

directed mutagenesis of the DNA coding sequence, in a way that will not negate its antigenic capability.

The invention also relates to at least partially purified antigenic *Bb* proteins or polypeptides which are capable of producing an *in vivo* immunogenic response when challenged with *Bb*. These will be useful in connection with vaccine development, and as antigen(s) in immunoassays for detection of *Bb* antibodies in biological fluids such as serum, seminal or vaginal fluids, urine, saliva, body exudates and the like.

In other aspects, the invention concerns recombinant vectors such as plasmids, phage or viruses, which comprise DNA segments in accordance with the invention, for use in replicating such sequences or even for the expression of encoded antigenic peptides or proteins. Vectors or plasmids may be used to transform a selected host cell. In preparing a suitable vector for transforming a cell, desired DNA segments from any of several *Bb* sources may be used, including genomic fragments, cDNA or synthetic DNA. In practice of the present invention, an expression vector may incorporate at least part of the DNA encoding one or more epitopic segments of the disclosed 30 kDa antigen.

Expression vectors may be constructed to include any of the DNA segments hereinabove disclosed. Such DNA might encode an antigenic protein specific for virulent strains of *Bb* or even hybridization probes for detecting *Bb* nucleic acids in samples. Longer or shorter DNA segments could be used, depending on the antigenic protein desired. Epitopic [illegible]

[illegible] expression vectors is possible including, for example, DNA segments encoding reporter gene products

useful for identification of heterologous gene products and/or resistance genes such as antibiotic resistance genes which may be useful in identifying transformed cells.

Recombinant vectors such as those described are particularly preferred for transforming bacterial host cells. Accordingly, a method is disclosed for preparing transformed bacterial host cells that includes generally the steps of selecting a suitable bacterial host cell, preparing a vector containing a desired DNA segment and transforming the selected bacterial host cell. Several types of bacterial host cells may be employed, including *Bb*, *E. coli*, *B. subtilus*, and the like as well as prokaryotic host cells.

Transformed cells may be selected using various techniques, including screening by differential hybridization, identification of fused reporter gene products, resistance markers, anti-antigen antibodies and the like. After identification of an appropriate clone, it may be selected and cultivated under conditions appropriate to the circumstances, as for example, conditions favoring expression or, when DNA is desired, replication conditions.

Another aspect of the invention involves the preparation of antibodies and vaccines from the antigenic 30 kDa protein or epitopic regions of that protein encoded by the disclosed DNA. The invention thus relates to one or more antibodies, monoclonal or polyclonal, that may be generated in response to the 30 kDa *Bb* protein or

has been obtained from other *Bb* antigens that are not associated with virulence. Previous work with several *Bb*

antigens isolated from both virulent and avirulent strains indicated low sensitivity when immunofluorescence and ELISA assays were employed, especially during early stages of infection.

In both immunodiagnostics and vaccine preparation, it is often possible and indeed more practical to prepare antigens from segments of a known immunogenic protein or polypeptide. Certain epitopic regions may be used to produce responses similar to those produced by the entire antigenic polypeptide. Potential antigenic or immunogenic regions may be identified by any of a number of approaches, *e.g.*, Jameson-Wolf or Kyte-Doolittle antigenicity analyses or Hopp and Woods (1981) hydrophobicity analysis (see, *e.g.*, Kyte and Doolittle, 1982, or U.S. Patent No. 4,554,101). Hydrophobicity analysis assigns average hydrophilicity values to each amino acid residue from these values average hydrophilicities can be calculated and regions of greatest hydrophilicity determined. Using one or more of these methods, regions of predicted antigenicity may be derived from the amino acid sequence assigned to, *e.g.*, the 30 kDa polypeptide. Proposed epitopic regions from the 30 kDa antigen include the sequences corresponding to positions 64-87, 106-114, 23-54, 128-133, 152-188 and 208-226.

It is contemplated that the antigens and immunogens of the invention will be useful in providing the basis for one or more assays to detect antibodies against *Bb*. Previous assays have used whole *Bb* as the antigen. Sera from normal individuals not exposed to *Bb* often contain

the diagnostic threshold of reactivity to avoid false positive reactions due to these cross-reactive antibodies

in normal sera. These adjustments may in turn decrease the sensitivity of the assay and lead to false negative reactions, particularly in the early stages of *Bb* infection. Assays using the disclosed 30 kDa protein or antigenic polypeptides thereof, are expected to give superior results both in sensitivity and selectivity when compared to assays that use whole *Bb* or even purified flagella in either an indirect ELISA or an antibody capture ELISA format. Western immunoblots based on reactions with such antigens (whole *Bb*, flagella and the like) have been difficult to interpret due to the presence of antibodies in sera from unexposed individuals. These antibodies cross react with *Bb* antigens, most particularly the 41 kDa flagellin and the 60 kDa common antigen protein. Generally, assays which use whole organisms or purified flagella tend to contain antigens with epitopes that will cross react with other bacterial antigens. For example, the N and C terminal regions of the *Bb* flagellin possess 52-55% sequence identity with the *Salmonella typhimurium* and *Bacillus subtilis* sequences (Wallich *et al.*, 1990), exemplifying the highly conserved nature of flagellin structure. The 60 kDa *Bb* protein is likewise 58% homologous with the *E. coli* protein (Shanafelt *et al.*, 1991). Such cross reactivity is not likely with the 30 kDa antigen, which is apparently unique to *Bb*.

It is further anticipated that a recombinant derived 30 kDa *Bb* protein will be particularly preferred for detecting *Bb* infections. Unexposed individuals should have a low reactivity to one or more epitopes of the 30 kDa protein thereby making it possible to use lower

Preferred immunoassays are contemplated as including various types of enzyme linked immunoassays (ELISAS), immunoblot techniques, and the like, known in the art. However, it will be readily appreciated that utility is not limited to such assays, and useful embodiments include RIAs and other nonenzyme linked antibody binding assays or procedures.

Yet another aspect of the invention is a method of detecting *Bb* nucleic acid in a sample. The presence of *Bb* nucleic acid in the sample may be indicated by the presence of the polypeptide products which it encodes. The method therefore includes detecting the presence of at least a portion of any of the polypeptides herein disclosed. Suitable detection methods include, for example, immunodetection reagents, PCR amplification, and hybridization.

Yet another aspect of the invention includes one or more primers capable of priming amplification of *Bb* DNA. Such primers are readily generated taking into account the base sequence of the DNA segment or deriving a base sequence from the amino acid sequence of a purified polypeptide encoded by the DNA. Primers are analogous to hybridization probes, but are generally relatively short DNA segments, usually about 7-20 nucleotides.

Methods of diagnosing Lyme disease are also included in the invention. In one embodiment, an antibody-based method includes obtaining a sample from a patient suspected of having Lyme disease, exposing that sample to one or more epitopes of the *Bb* protein which is encoded

indicative of the presence of Lyme disease. Typical samples obtainable from a patient include human serum,

plasma, whole blood, cerebrospinal fluid, seminal or vaginal fluids, exudates and the like.

Several variations of antibody-based methods are contemplated for development; for example, an indirect ELISA using the 30K protein or other *Bb* proteins as an antigen. The 30K protein may be produced in large quantities by recombinant DNA vectors already disclosed and purified. Optimal concentration of the antigen could be determined by checker board titration and diagnostic potential of the 30 kDa protein assay examined further by testing serum from mice at different stages of infection and infected with different strains of *Bb*. These results could indicate the relative time course for sera conversion for each of the assays and would also show whether infection with different strains causes variation in anti-30 kDa protein titers.

Likewise, reactive epitopes of the 30 kDa polypeptide are contemplated as useful either as antigens in an ELISA assay or to inhibit the reaction of antibodies toward intact 30 kDa protein bound to a well. Epitopic peptides could be generated by recombinant DNA techniques previously disclosed or by synthesis of peptides from individual amino acids. In either case, reaction with a given peptide would indicate presence of antibodies directed against more epitopes. In addition to its diagnostic potential, this method is seen as being particularly effective in characterizing monoclonal antibodies against the 30 kDa protein and other virulence associated proteins.

The invention also [illegible]

embodiments, such antibodies lack cross reactivity with antigens found in other bacteria. Monoclonal antibodies

against the 30 kDa protein and other virulence associated proteins are generated by using hybridomas which can be produced and screened. Proteins produced by *Bb* or recombinant DNA vectors and purified by two-dimensional electrophoresis or other methods could be used for immunization of animal models such as BALB/C mice. Selection of reactive clones is carried out with a typical ELISA assay using the immunizing protein as antigen. Western immunoblots could also be used in a screening or confirmatory assay.

Such monoclonals are envisioned as useful in several respects including (1) detection of *Bb* in tissues or body fluids by immunofluorescence, enzyme immunoreactions, such as immunoperoxidase staining of tissue sections, avidin-biotin indicator enzyme immunoassays, or other techniques, (2) rapid screening of *Bb* strains and clones as well as *E. coli* recombinants for expression of the protein, (3) determination of structural locations of proteins by immuno electron microscopy, (4) identification of reactive epitopes using a peptide library, (5) demonstration of bacteriocidal activity *in vitro* in combination with compliment and selection of protein deficient mutants, (6) assessment of immunoprotective activity by passive immunization, (7) use to study host cell interactions by inhibition of adherence or penetration or by enhancement or engulfment and killing by phagocytic cells, and (8) possible use for epidemiological studies particularly in studying variation of *Bb* strains in expression of proteins or protein sequences.

protein 30 kDa or a protein or peptide which includes an epitope thereof, together with means for detecting a

specific immunoreaction between an antibody and its corresponding antigen. Examples of suitable means include labels attached directly to the antigen or antibody, a secondary antibody having specificity for human Ig, or protein A or protein G. Alternatively, avidin-biotin mediated *Staphylococcus aureus* binding could be used. For example, the monoclonal antibody may be biotinylated so as to react with avidin complexed with an enzyme or fluorescent compound.

A particular kit embodiment of the invention concerns detection of antibodies against the described *Bb* 30 kDa antigen, epitopes thereof as represented by portions of the amino acid sequences, or closely related proteins or peptides, such as epitopes associated with other virulence-associated proteins detected by comparison of low-passage, virulent and high-passage, avirulent strains of *Bb*. The antigen for the kit(s) consists of the *Bb* 30 kDa protein or portions thereof produced by a recombinant DNA vector in *E. coli* or another bacterial or nonbacterial host. Alternatively, the antigen may be purified directly from *Bb* or manufactured as a synthetic peptide. Samples for the assays may be body fluids or other tissue samples from humans or animals. The presence of reactive antibodies in the samples may be demonstrated by antibody binding to antigen followed by detection of the antibody-antigen complex by any of a number of methods, including ELISA, RIA, fluorescence, agglutination or precipitation reactions, nephelometry, or any of these assays using avidin-biotin reactions. The degree of reactivity may be assessed by comparison to control samples, and the degree

e.g., to determine the efficacy of therapy.

In still further embodiments, the invention contemplates a kit for the detection of *Bb* nucleic acids in the sample, wherein the kit includes one or more nucleic acid probes specific for the 30 kDa gene, together with means for detecting a specific hybridization between such a probe and *Bb* nucleic acid, such as an associated label.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A through 1B shows a comparison of the 2-dimensional protein electrophoresis patterns of high-infectivity clone Sh2-5A5 (Fig. 1A) and low-infectivity clone Sh2-5A1 (Fig. 1B) of *B. burgdorferi*. The locations of the 41 kDa flagellin protein (Fla), the 34 kDa outer surface protein B (OspB), and the 31 kDa outer surface protein A (OspA) are indicated. The patterns are remarkably similar, except for the presence of a 38.8 kDa protein in the Sh2-5A5 pattern (arrow).

Figures 2A, 2B, 2C, 2D, 2E, and 2F shows an expanded view of the region containing the 38.8 kDa protein. Patterns in the region between Fla and OspB are shown for the high-infectivity clones Sh2 5A5, 5A4, and 5A3 (Figure 2A, 2B and 2C, respectively), and the low-infectivity clones Sh2 5A1, 5A2, and 5A3 (Figure 2D, 2E, and 2F, respectively). The location of the 38.8 kDa protein is indicated; a distinct spot is visible in Figure 2A and 2B, and the spot appears to be fused with the Fla spot in

low-infectivity clones (Figure 2D, 2E and 2F).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to the utility of *Bb* associated antigenic proteins as diagnostic or preventive tools in Lyme disease. Proteins have been identified as associated only with virulent isolates of *Bb*, providing a basis for several types of diagnostic tests for Lyme disease, including immunodiagnostic and nucleic acid identification, such as those based on amplification procedures.

The DNA of the present invention was isolated from the bacteria *Borrelia burgdorferi* hereafter designated as *Bb*. The microorganism is a spiral-shaped organism approximately 0.2 micron in diameter and ranging in length from about 10-30 microns. Like other spirochetes, it possesses an inner membrane, a thin peptidoglycan layer, an outer membrane, and periplasmic flagella which lie between the inner and outer membranes. *Bb* is obligate parasite found only in association with infected animals and arthropod vectors in endemic areas. *Bb*-like organisms have also been identified in birds raising the possibility that birds could also serve as an animal reservoir. While some *Bb* isolates have been cloned, most isolates have not been cloned and most likely represent mixtures of different variants even at the time of culture origination.

Bb has similarities with other relapsing fever organisms such as *B. hermsii*. *Bb* has a single chromosome with two unusual features, linear conformation and small size (approximately 900 kilobase pairs). Fresh isolates of *Bb* contain up to four linear plasmids and six circular

study only two of thirteen strains had similar plasmid profiles. Some plasmids are lost during *in vitro* passage

which may correlate with loss of virulence. Outer surface proteins OspA and OspB are encoded on the 49 kbp linear plasmid. A 30K virulence-associated protein discovered by the inventors is encoded on a 38 kbp plasmid. Generally, the functions of the *Bb* plasmids are unknown.

It will be recognized that there is a high degree of variability among *Bb*, especially among *Bb* isolates and depending on the number of *in vitro* passages to which the cultures have been subjected. Generally there are two types of variation that occur among *Bb* strains; (1) natural heterogeneity present in fresh isolates of *Bb*, and (2) the artificial changes resulting from *in vitro* culture. In terms of natural heterogeneity, there is now evidence that at least two distinct populations of *Bb* exist based on the chromosomal DNA sequences. Primers directed to these DNA sequences indicate two major classes of DNA, one specific to North American *Bb* isolates, and the other specific for most European isolates. Additionally, primers have been found which recognize all *Bb* strains. There is significant variability among strains from all geographic locations in terms of plasmid content as well as protein profile, particularly in terms of the molecular weights of the OspA and OspB proteins among European isolates.

In vitro passage of *Bb* results in loss of plasmids and an apparent concomitant loss of infectivity and virulence in animal hosts. Typically these changes occur within the first 10-17 passages *in vitro*. It is likely that non-infectious clonal variants begin to occur as

As used herein, *Bb* isolates have been referred to as "low-passage" or "high-passage". *Bb* cultures were grown in culture tubes and passaged though various numbers of subcultures. Generally, low-passage isolates underwent 10 or less subcultures while high-passage isolates were cultured often up through 100 passages.

A 30 kDa protein has been identified in low-passage, virulent strains of *Bb*, but is absent or underexpressed in isogenic high-passage, avirulent strains. Because of the instability of *Bb* during *in vitro* culture, it was important that a low passage number (<10) strain was available for each isolate and that the virulence of the strain was documented. Virulence was confirmed by inoculating 10^4 organisms into the backs of 3-week old mice known to be highly susceptible to *Bb* infection. Using this protocol, low passage, virulent strains of *Bb* were found to express a major protein not found in avirulent, high passage strains.

Detection of *Bb* proteins utilized a modification of two dimensional gel electrophoresis. By employing relatively short run times on the gel during the first dimension run, about 4 hrs in comparison to up to 16 hrs in usual isoelectric focusing, protein migration was not at equilibrium, allowing basic proteins especially to focus on the gel and be resolved. The second dimension used was a polyacrylamide gradient SDS-PAGE. Molecular weights were estimated by running molecular weight markers with the solubilized *Bb* proteins on the gel. This method permitted identification of several polypeptides unique to low passage, virulent *Bb* strains, implying a possible correlation with virulence or infectivity.

As used herein, DNA segments encoding a polypeptide identified by its approximate molecular weight is referred to by the corresponding number and the letter "K". Thus, as used herein, 30K represents the gene encoding a 30 kDa polypeptide. An alternate designation, also used herein, for the 30K gene is lpa30.

In order to identify DNA segments encoding the 30 kDa protein, purified protein was isolated from a low-passage, virulent *Bb* strain by preparative two dimensional electrophoresis for subsequent use in amino acid sequencing. Initial studies indicated that the N-terminus was blocked. After cyanogen bromide cleavage, separation of the resulting peptide fragments by electrophoresis, and transfer of the peptides to polyvinylene diffusible membranes, sequence analysis was performed using standard sequencing techniques (Matsudaira, 1987). Two of the peptide fragments had overlapping sequences. A 16 amino acid sequence was identified. Codons for the amino acid sequence were selected by reverse translation based on (1) conclusion that codons containing A or T were favored and (2) knowledge of published DNA sequences for several *Bb* proteins. A choice favoring A or T containing codons was based on the observation that the G + C content of *Bb* is only 28-35%. A 33 nucleotide segment was synthesized.

The 33 residue oligonucleotide probe was used as a probe to identify DNA encoding the 30 kDa protein isolated from low-passage, virulent *Bb*. Bacterial cells from low-passage cultures of *Bb* strain B31 were precipitated, lysed and extracted with phenol/chloroform.

pUC19 and a library was generated. Clones were isolated, subjected to agarose gel, Southern blot and then

hybridized with the radiolabeled oligonucleotide probe. Hybridization occurred with the 87th clone tested.

The oligonucleotide probe hybridized with a 1.0 kb DNA fragment that was isolated by agarose gel electrophoresis. The single clone from which the 1.0 kb DNA was isolated contained a plasmid with a 950 bp insert to which the prepared oligonucleotide probe bound with a 500-600 bp *Pst*I fragment within the insert. The DNA sequence was determined and a deduced amino acid sequence identified.

The nucleotide probe was used to determine whether the 30K gene was present and expressed in low-passage isolates of *Bb* from different geographic locations. The probe hybridized with plasmids with apparent sizes from low-passage isolates from HB19 (Connecticut), pKa I (Munich, Germany), and G25 (Sweden). The 30K gene was thus shown to be present in strains from distant geographic regions.

A genomic library has also been prepared from *Bb* strain HB19. This could readily be done for other strains of *Bb* and will be useful in preparing clones for isolating DNA encoding proteins similar or identical with the 30 kDa protein isolated from strain B31. For example, DNA suspected of encoding a virulence associated protein may be genetically transferred, that is, by transfer of DNA into *Bb* via a plasmid with demonstration of production of particular protein expression. Demonstration of virulence using clonal variation would then provide evidence of a causal relationship between

groups, usually 3-4, of C3H/HeN mice with ~10^6 organisms. The mice were then observed for joint swelling, bled and

sacrificed after 3 weeks. Arthritogenesis, seroconversion and positive cultures from heart, bladder, or other organs provided evidence of virulence. This mouse model appears at present to be the best animal model for Lyme borreliosis (Barthold *et al.*, 1990). Only a few organisms (~20) are required to establish infection intradermally, and the mice exhibit chronic, systemic spirochetosis and uniform arthritogenesis and carditis. Other animal models such as rats, hamsters, rabbits, may require high dosages for infection and have relatively mild pathology (Moody *et al.*, 1990). Immunocompromised states (neonatal infection, gamma irradiation) may be necessary to establish infection. Furthermore, it has been shown (Fikrig *et al.*, 1990) that the mouse model may be used to demonstrate both passive and active immunity. C3H/HeN mice may be protected from infection with the N40 strain of *Bb* by injection with anti-OspA monoclonal antibodies and anti-N40 antiserum or immunization with recombinant OspA protein (Fikrig *et al.*, 1990).

Antigenicity of the 30K protein was determined. Antiserum collected from rabbits injected with the protein was shown to react with the 30K protein, as detected on 2-dimensional gel electrophoresis immunoblots of low-passage *Bb* B31. No reactive spots were detected in normal rabbit serum. This result should lead to straightforward production of monoclonal antibodies reactive with the 30K polypeptide and possibly other virulence-associated proteins. Antibodies could be produced in mice and used for screening strains for protein expression, for determining structural location and for examining bactericidal activity of antibodies

disease may be developed using any of the 30K protein or its epitopes, the corresponding DNA encoding the protein,

functionally similar proteins and their epitopes, or by detection of the appropriate mRNA. An indirect ELISA assay is set forth in Example 9 and could be used with the 30 kDa protein or other antigenic *Bb* proteins, such as the 20K protein. These methods are similar in principle to those previously described (Magnarelli *et al.*, 1989; Magnarelli *et al.*, 1984; and Craft *et al.*, 1984). Reactive epitopes representing portions of the 30 kDa protein sequence could be utilized in an analogous manner.

Another promising assay is the microcapsule agglutination technique (MCAT) (Arimitsu *et al.*, 1991). In this procedure, microscopic polystyrene beads are coated with *Bb* antigen and incubated with dilutions of patient serum. After overnight incubation at 4°C, the agglutination patterns are determined. Using whole *Bb* as antigen, the MCAT has been shown to be highly discriminatory between Lyme disease patients and healthy individuals, with little overlap in agglutination titer, although false positive reactions have been obtained with rheumatoid arthritis patients (Anderson *et al.*, 1988; and *Centers Dis. Control*, 1988) and leptospirosis samples (Barbour, 1988; and *Centers for Disease Control*, 1989). An assay using 30 kDa protein alone or in combination with other antigens such as the 94K, 30K and 21K antigens should be feasible. Such combination may increase sensitivity of the assay.

The invention has disclosed a DNA segment encoding an antigenic protein that is apparently associated only with virulent strains of *Bb*. Detection of that DNA or

portions of the gene. A particular method utilizes PCR amplification, using any of a number of primers that

could be prepared from knowledge of the nucleic acid sequence. Generally, such primers are relatively short, *e.g.*, 7-28 base pairs in length, and may be derived from the respective sense or anti-sense strands of the disclosed DNA segment. Synthesis of these primers may utilize standard phosphoramidite chemistry (Beaucage *et al.*, 1981).

Part of the present invention contemplates vaccine preparation and use. General concepts related to methods of preparation and use are discussed as applicable to preparations and formulations with the disclosed 30 kDa antigen, its epitopes and subfragments thereof.

Vaccine Preparation and Use

Preparation of vaccines which contain peptide sequences as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, all incorporated herein by reference. Typically, such vaccines are prepared as injectables either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10-95% of active ingredient, preferably 25-70%.

The proteins may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include acid addition salts (formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

quantity to be administered depends on the subject to be treated, including, e.g., the capacity of the

individual's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination. Suitable regimes for initial administration and booster shots are also variable but are typified by an initial administration followed by subsequent inoculations or other administrations.

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the size of the host.

Various methods of achieving adjuvant effect for the vaccine include use of agents such as aluminum hydroxide or phosphate (alum), commonly used as 0.05 to 0.1 percent solution in phosphate buffered saline, admixture with synthetic polymers of sugars (Carbopol) used as 0.25 percent solution, aggregation of the protein in the vaccine by heat treatment with temperatures ranging between 70° to 101°C for 30 second to 2 minute periods respectively. Aggregation by reactivating with pepsin treated (Fab) antibodies to albumin, mixture with bacterial cells such as *C. parvum* or endotoxins or lipopolysaccharide components of gram-negative bacteria,

as a block substitute may also be employed.

In many instances, it will be desirable to have multiple administrations of the vaccine, usually not exceeding six vaccinations, more usually not exceeding four vaccinations and preferably one or more, usually at least about three vaccinations. The vaccinations will normally be at from two to twelve week intervals, more usually from three to five week intervals. Periodic boosters at intervals of 1-5 years, usually three years, will be desirable to maintain protective levels of the antibodies. The course of the immunization may be followed by assays for antibodies for the supernatant antigens. The assays may be performed by labeling with conventional labels, such as radionuclides, enzymes, fluorescers, and the like. These techniques are well known and may be found in a wide variety of patents, such as U.S. Patent Nos. 3,791,932; 4,174,384 and 3,949,064, as illustrative of these types of assays.

The invention also contemplates the use of disclosed nucleic acid segments in the construction of expression vectors or plasmids and use in host cells. The following is a general discussion relating to such use and the particular considerations in practicing this aspect of the invention.

Host Cell Cultures and Vectors

In general, of course, prokaryotes are preferred for the initial cloning of DNA sequences and constructing the vectors useful in the invention. For example, in addition to the particular strains mentioned in the more specific disclosure below, one may mention by way of example strains such as E. coli K12 strain 294 (ATCC N

Prokaryotes are also preferred for expression. The aforementioned strains, as well as *E. coli* W3110 (F-, lambda-, prototrophic, ATCC No. 273325), bacilli such as *Bacillus subtilus*, or other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcesans*, and various *Pseudomonas* species may be used.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (see, *e.g.*, Bolivar *et al.*, 1977). The pBR322 plasmid contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the microorganism for expression.

Those promoters most commonly used in recombinant DNA construction include the B-lactamase (penicillinase) and lactose promoter systems (Chang *et al.*, 1978; Itakura *et al.*, 1977; Goeddel *et al.*, 1979) and a tryptophan (trp) promoter system (Goeddel *et al.*, 1979; EPO Appl. Publ. No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors

addition of another promoter by artificial means.

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used. *Saccharomyces cerevisiase*, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in *Saccharomyces*, the plasmid YRp7, for example, is commonly used (Stinchcomb *et al.*, 1979; Kingsman *et al.*, 1979; Tschemper *et al.*, 1980). This plasmid already contains the *trp*1 gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, 1977). The presence of the *trp*1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzman *et al.*, 1980) or other glycolytic enzymes (Hess *et al.*, 1968; Holland *et al.*, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination.

Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose

utilization. Any plasmid vector containing a yeast compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (Tissue Culture, 1973). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 293 and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers *et al.*, 1978). Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the *Hind*III site toward the *Bgl*I

with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (*e.g.*, Polyoma, Adeno, VSV, BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

Also contemplated within the scope of the present invention is the use of the disclosed DNA as a hybridization probe. While particular examples are provided to illustrate such use, the following provides general background for hybridization applications taking advantage of the disclosed nucleic acid sequences of the invention.

Nucleic Acid Hybridization Embodiments

As mentioned, in certain aspects, the DNA sequence information provided by the invention allows for the preparation of relatively short DNA (or RNA) sequences having the ability to specifically hybridize to *B. burgdorferi* gene sequences. In these aspects, nucleic acid probes of an appropriate length are prepared based on a consideration of the sequence or derived from flanking regions of these genes. The ability of such nucleic acid probes to specifically hybridize to the *B. burgdorferi* gene sequences lend them particular utility in a variety of embodiments. Most importantly, the probes can be used in a variety of diagnostic assays for detecting the presence of pathogenic organisms in a given sample. However, either uses are envisioned, including the use of the sequence information

To provide certain of the advantages in accordance with the invention, the preferred nucleic acid sequence employed for hybridization studies or assays includes sequences that are complementary to at least a 10 to 40, or so, nucleotide stretch of the selected sequence. A size of at least 10 nucleotides in length helps to ensure that the fragment will be of sufficient length to form a duplex molecule that is both stable and selective. Molecules having complementary sequences over stretches greater than 10 bases in length are generally preferred, though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. Thus, one will generally prefer to design nucleic acid molecules having gene-complementary stretches of 15 to 20 nucleotides, or even longer where desired. Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR technology of U.S. Patent 4,603,102, or by introducing selected sequences into recombinant vectors for recombinant production.

The present invention will find particular utility as the basis for diagnostic hybridization assays for detecting *Bb*-specific RNA or DNA in clinical samples. Exemplary clinical samples that can be used in the diagnosis of infections are thus any samples which could possibly include nucleic acid, including samples from tissue, blood serum, urine or the like. A variety of tissue hybridization techniques and systems are known which can be used in connection with the hybridization

Accordingly, the nucleotide sequences of the invention are important for their ability to selectively form duplex molecules with complementary stretches of *B. burgdorferi* gene segments. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degree of selectivity of the probe toward the target sequence. For applications requiring a high degree of selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, for example, one will select relatively low salt and/or high temperature conditions, such as provided by 0.02M-0.15M NaCl at temperatures of 50°C to 70°C. These conditions are particularly selective, and tolerate little, if any, mismatch between the probe and the template or target strand.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template, less stringent hybridization conditions are called for in order to allow formation of the heteroduplex. In these circumstances, one would desire to employ conditions such as 0.15 M-0.9 M salt, at temperatures ranging from 20°C to 55°C. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

for determining hybridization. A wide variety of appropriate indicator means are known in the art,

including radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred diagnostic embodiments, one will likely desire to employ an enzyme tag such as alkaline phosphatase or peroxidase, instead of radioactive or other environmentally undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known which can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with pathogen nucleic acid-containing samples. Luminescent substrates, which give off light upon enzymatic degradation, could also be employed and may provide increased sensitivity.

In general, it is envisioned that the hybridization probes described herein will be useful both as reagents in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) from suspected clinical samples, such as exudates, body fluids (*e.g.*, amniotic fluid cerebrospinal fluid) or even tissues, is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required (depending, for example, on the G+C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe, etc.). Following washing of the hybridized surface so as to remove nonspecifically bound probe molecules, specific

The present invention has addressed the cloning of nucleic acids encoding certain antigenic polypeptides

related to a 30 kDa protein. Identification of virulence-associated proteins in addition to the 30 kDa antigenic protein should be possible using methods analogous to those disclosed herein. One method would be to produce a cDNA library using mRNA obtained from low-passage isolates. Although the production of cDNA libraries from bacteria is not commonly done because of the usual absence of poly-A tails on prokaryotic messages, a cDNA library has been constructed from *Borrelia hermsii* mRNA. The technique involves use of random primers and reverse transcriptase to produce the initial cDNA. From that point linkers are attached and the inserts cloned into a suitable plasmid or bacteriophage vector. This technique lends itself also to use with "subtraction" techniques. In this way, DNA from a high-passage, non-infectious isogenic isolate can be used to hybridize transcripts common to high and low-passage isolates out of the mRNA.

A method of preparing variants of the 30 kDa antigen is site-directed mutagenesis. This technique is useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence

with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art as exemplified by publications (Adelman *et al.*, 1983). As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage (Messing *et al.*, 1981). These phage are readily commercially available and their use is generally well known to those skilled in the art.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector which includes within its sequence a DNA sequence which encodes the 30 kDa antigen. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example by the method of Crea *et al.* (1978). This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

not meant to be limiting as there are other ways in which sequence variants of the 30K gene may be obtained. For

example, recombinant vectors encoding the desired 30K gene may be treated with mutagenic agents to obtain sequence variants (see, *e.g.*, a method described by Eichenlaub, 1979) for the mutagenesis of plasmid DNA using hydroxylamine.

The following examples are intended to illustrate the practice of the present invention and are not intended to be limiting. Although the invention is demonstrated with a 30 kDa protein from *Bb*, other antigenic proteins unique to virulent strains might be used in a similar fashion. The proteins identified and the encoding DNA are clearly useful in developing selective and sensitive assays for Lyme disease and in potentially distinguishing virulent infections of *Bb* in humans.

EXAMPLE 1

The present example illustrates the differences in polypeptides produced in low-passage, virulent strains of *Bb* and those produced in isogenic, high passage, avirulent strains. This was intended to identify proteins essential for infectivity and virulence of *Bb* in mammalian hosts. Because different isolates are likely to possess differences unrelated to virulence, comparisons were performed between well-defined, low passage strains and the same strains following prolonged *in vitro* passage.

One of the initial problems in separating *Bb* proteins was the loss of several major proteins, including OspA and OspB, when [illegible] modification of isoelectric focusing called non equilibrium pH gradient electrophoresis (NEPHGE)

(O'Farrell *et al.*, 1977) was successful in resolving all major *Bb* polypeptides. This technique utilizes shorter run times so that all polypeptides are retained in the gel pattern.

NEPHGE of Virulent and Avirulent Strains of *Bp*

The B31 strain of *Bb* was passaged in BSKII medium and incubated at 34°C until late log phase (7-10 days). Low passage was less than 10 passages while high passage was >100 *in vitro* passages. For protein analysis, *Bb* from late log phase cultures was washed three times by centrifugation and gently resuspended in phosphate-buffered saline and stored at -70°C. Organisms (2×10^8 per gel) were sonically disrupted, suspended in solubilization buffer and subjected to NEPHGE in 3 mm tube gels. NEPHGE differed from standard 2-dimensional gel electrophoresis primarily in the duration of electrophoresis. Run time was 4 hrs at 400 volts, as opposed to 16 hrs for isoelectric focusing (IEF). Since protein migration was not at equilibrium, many proteins not focused on standard IEF were resolved. The second dimension gel consisted of SDS-PAGE with an 8 to 20 percent polyacrylamide gradient to enhance separation. Single dimension lanes containing solubilized *Bb* and molecular weight standards (BioRad, Richmond, CA) were placed on either end of the tube gel. Polypeptides were visualized by silver staining; alternatively, the 2-dimensional gel electrophoresis pattern was transferred to a PVDF membrane for immunoblot analysis (Matsudaira, 1987).

[illegible] polypeptides gel patterns were compared visually to detect qualitative similarities and differences. Although not employed for measurements [illegible]

is contemplated that quantitative analysis could be carried out using a Visage 2000 Image Analysis system (BioImage, Ann Arbor, MI). The instrument densitometer/computer system is used to scan, store, and interpret an image array of 1024 x 1024 pixels (0.18 mm^2/pixel). Two-dimensional gel electrophoresis (2DGE) spot identification, alignment and quantification data are collected and analyzed utilizing a Visage Image Analysis software package.

Low passage and high passage isolates of the B31 strain of *Bb* were subjected to less than 5 and greater than 100 *in vitro* passages, respectively. Over 100 spots were detected by silver staining in each pattern, but the 2D pattern was dominated by a few major structural proteins, as was also the case with *T. pallidum*. Streaking of the major spots toward the origin of migration (the acid end) occurred due to the non-equilibrium nature of the NEPHGE separation. OspA, OspB, and the 41K flagellin were identified by M_r and by their reactivities with monoclonal antibodies. The MoAb H68 also reacted with a series of 20K spots in the low passage isolate, indicating the presence of shared epitopes between OspB and the 20K polypeptide. Rabbit antiserum against a 24K polypeptide reacted with a major spot in low passage B31; this anti-serum also reacted with a basic 35K polypeptide.

The most noticeable difference low passage and high passage B31 was the presence of a major, acidic polypeptide with an M_r of 30,500 in the low passage isolet. This polypeptide, called the 30K protein, lies

polypeptide in the high passage B31. The 20K polypeptide reactive with MoAb H68 was also absent from high passage

B31. The 24K protein was expressed in smaller quantities, whereas the basic 35K polypeptide which reacted with the anti-24K anti-serum was not detectable in the high passage isolate. Similar results were obtained with well-defined low and high passage variants of the North American human blood isolate HB19 (not shown).

EXAMPLE 2

The observation of a polypeptide found in low passage *Bb* but not detectable in high passage *Bb* strain B31 led to efforts to isolate and characterize this apparently unique protein. The protein, as indicated in Example 1, was a major, acidic protein that was suspected of being associated with virulence.

Purification and Partial Sequence Determination of the 30K Protein

Approximately 5 micrograms of the 30K protein was purified from low passage B31 by large scale two-dimensional electrophoresis, as developed for the purification of *T. pallidum* polypeptides (Norris *et al.*, 1988). 10^{10} *Bb* were solubilized, loaded onto 24 tube gels, and subjected to NEPHGE. The tube gels were stained with Coomassie blue G, and the protein band corresponding to the 30K protein, as determined by comparing to a 2D gel pattern, was carefully sliced out. The excised bands were equilibrated with SDS PAGE buffer, and electrophoresed together on a single SDS PAGE gel. The Coomassie blue stain spots representing purified 30K

cleave the protein internally at methionine residues. The resulting fragments were separated on a 20%

acrylamide SDS page gel and transferred to a PVDF membrane for sequence analysis (Matsudaira, 1987). N-terminal sequence was obtained from two prominent peptides which had similar M_r's. These turned out to be overlapping peptides due to the presence of two methionine residues separated by only five intervening amino acids.

EXAMPLE 3

After determining partial amino acid sequence of a *Bb* protein that appeared to be unique to virulent, low passage strains of *Bb* strain B31, see Example 2, it was desired to develop a means to detect DNA sequences in B31 and other strains. This was achieved by synthesizing an oligonucleotide probe, derived from the amino acid sequence determined from the 30 kDa protein using the most common codon usage observed in the *Bb* genes that had already been sequenced (see Example 2).

DNA Hybridization

The nucleotide sequence is derived from the 30K amino acid sequence using the most common codon usage observed in the *Bb* genes sequenced. G + C content of *Bb* is 28-30%. Thus codons containing A or T are highly favored.

The radiolabeled nucleotide was used as a probe to determine whether complementary sequences were present in Southern blots of whole, genomic *Bb* DNA from low passage and high passage B31. Under conditions of low

The 30K encoding sequence was localized to a plasmid with an apparent size of 38 kilobase pairs, using DNA hybridization of the oligonucleotide to Southern blots of *Bb* plasmid preparations. This plasmid appeared to be linear; high passage strains lacking the 30K protein were also missing the 38 kbp plasmid. The 30K oligonucleotide did not hybridize to Southern blots of plasmid preparations from these strains. Northern blots confirmed that an mRNA species that hybridized with the above probe was expressed in low passage B31 strains but not in high passage strains. The oligonucleotide was also found to hybridize to similar plasmids in several other low passage *Bb* isolates, including HB-19 (human blood isolate, U.S.), PB1 and Munich86 (human cerebrospinal fluid isolates, Germany), G25 (Sweden), and Veery (U.S. bird isolate). No corresponding plasmid was found in high passage strains of *Bb* or in *Borrelia hermsii*.

EXAMPLE 4

The hybridization of the oligonucleotide probe of Example 2 with a *Hind*III DNA fragment associated with low passage *Bb* strain B31 but not with high passage B31, strongly implicated the DNA as encoding the 30 kDa polypeptide typically associated with low passage *Bb* strains. Therefore, molecular cloning of the DNA was undertaken.

Molecular Cloning of the 30K Gene

To facilitate the cloning of the 30K gene, *Hind*III

into the plasmid pUC19 which had been cleaved with *Hind*III and dephosphorylated. *E. coli* strain Jm109 was

transformed with the recombinant plasmids, and the resulting recombinants were screened by hybridization with the oligonucleotide under conditions of low stringency. After repeated screening, a single clone (pTB087A) that hybridized to the oligonucleotide was isolated. TB087A contained a 950-bp *Bb* DNA insert, and the oligonucleotide was bound to a 500-600 *Pst*I fragment within this insert. 80% of the 30K gene and an additional 300 base pairs of upstream, untranslated DNA was obtained. An additional 1.5 kb *Pst*I fragment containing the remainder of the 30K gene was cloned into pUC19.

Dideoxynucleotide sequencing of clone Tbo87 demonstrated the presence of an extended open reading frame encoding 257 amino acids. Typical -35 and -10 σ70 recognition sites and Shine Delgarno ribosome binding site sequences were found upstream of the presumed start codon. Several additional -35 and -10 sequences were found further upstream, possibly indicating unusual transcriptional regulation mechanisms.

The N-terminal region of the deduced amino acid sequence was typical of the signal peptides of bacterial lipoproteins. The N-terminal methionine was followed by a cluster of lysine residues, a hydrophobic region and a signal peptidase 2 (SP2) recognition sequence. The latter sequence, differed somewhat from the consensus SP2 recognition sequence found in most bacteria, but closely resembled the cleavage sequence of the variable major proteins Vmp7 and Vmp21 of *B. hermsii*. These variable surface antigens of relapsing fever organisms have been shown to be lipoproteins (Burman [illegible]

membrane and is attached to the cytoplasmic membrane

and/or outer membranes via fatty acids associated with an N-terminal cysteinyl residue.

The Tbo87 clone containing 30K gene was identified by hybridization with an oligonucleotide sequence based on a CNBr fragment amino acid sequence. A sequence corresponding exactly to the CNBr cleavage fragment was identified as residues 119-129 in the deduced amino acid sequence, confirming the identity of the gene. The oligonucleotide used for screening was identical at 30 of 33 positions.

Analysis of the secondary structure of the gene was conducted using the method of Garnier *et al.*, 1978. An alpha helical structure for over 90% of the sequence was predicted, glycosylation sites were also indicated.

A restriction map of the cloned DNA indicates the location and orientation of the 30K gene. The region between the two *Hind*III sites on the left represents clone Tbo87. The cloned region between the *Pst*I sites represents the region cloned to obtain the remainder of the 30K sequence.

EXAMPLE 5

The presence of the 30K gene in low passage, virulent strains of *Bb* obtained from different geographic locations was examined, with the intention of determining the generality of existence of this gene and its associated 30 kDa associated gene product. All strains tested indicated the presence of this gene, indicating a

Presence of the 30K gene In Virulent *B. burgdorferi* Isolates

The 30K oligonucleotide described in Example 3 was hybridized with plasmids having apparent sizes of 38-40 kilobase from several low passage isolates including HB19 (Connecticut), PB1, Munich 86, and PKA1 (Germany), and G25 (Sweden). Plasmids were isolated, purified, run on an agarose gel followed by Southern blot and hybridized with the oligonucleotide probe prepared as described by Barbour, (1988). The 30K gene was thus shown to be present in strains from a variety of geographic regions. The 30K protein was expressed by HB19 in a quantity similar to that found in B31.

EXAMPLE 6

Genomic DNA libraries may be prepared from several *Bb* strains and DNA sequences compared. This would assist in identifying other virulence specific antigens and in determining the underlying molecular basis of virulence associated with *Bb*. The following example illustrates preparation of a genomic library from *Bb*, strain HB19.

Genomic Library From a Low Passage Infectious Isolate of *B. burgdorferi*

A low passage isolate of a human blood isolate (Steere *et al.*, 1983) was used to prepare a genomic library. This isolate had been shown to be infectious for rats and mice. This was confirmed by successfully infecting scid mice with this isolate. Four mice were inoculated intraperitoneally with 10^7

euthanasia at three weeks. All mice were shown to be spirochetemic at each culture and at autopsy. When high

passage isolate of the HB19 was inoculated at the same inoculum into scid mice, none of the mice showed evidence of infection after culture of blood or at autopsy.

The low passage HB19 isolate was grown in BSK II medium. Total DNA was extracted using a phenol/chloroform extraction and standard techniques (Hinnebusch *et al.*, 1990). A genomic DNA library was prepared using lambda cloning factor FIXII (Strategene, La Jolla, CA). Total DNA from low passage HB19 was partially digested with *Sau*3A and ligated with lambda arms with partially filled *Xho*II ends. The *spi* selection provided for the cloning of 15-23 kbp inserts of *borrelia* DNA in the vector; 3.9 x 10^6 primary plaques on P2 lysogen were obtained. The vector alone produced no plaques when plated on a P2 selective host.

Total DNA content of *B. burgdorferi* was approximately 1100 kilobases. The size of the library was considered sufficient on a statistical basis to be representative of the entire genome. The library was screened with probes for the genes for OspA, OspB (Bergström *et al.*, 1989) and for the flagellin protein (Sadziene *et al.*, 1991). Clones containing hybridizing sequences for each of these probes were present in the phage library at a frequency between 10^{-2} and 10^{-3}, leading to the conclusion that the phage library was likely representative of the genome.

EXAMPLE 7

It was shown in Example 1 that a major 30 kDa

useful in developing specific antibodies to *Bb* for diagnostic purposes and vaccine development. This

example demonstrates that the 30 kDa polypeptide isolated from low passage, virulent *Bb* strain induces an antibody response in rabbits.

Antigenicity of the 30K Protein

Rabbits were immunized by injections at bi-weekly intervals with the 30 kDa protein obtained from *Bb*, strain B31. Each rabbit was injected with 5 μg antigen (1.7 μg/kg) in distilled water. The antigen was purified by two-dimensional electrophoresis and emulsified in complete Freunds adjuvant (first injection) and incomplete Freunds adjuvant (subsequent injections). Anti-serum from this rabbit reacted with a spot corresponding to the 30 kDa protein from *Bb*, strain B31, in two-dimensional gel electrophoresis immunoblots of low passage B31, whereas normal rabbit serum did not react. The reactive spot was not detected in 2DGE immunoblots of high passage B31. Both the anti-30 kDa anti-serum and normal rabbits serum possessed background reactivity with several *B. burgdorferi* polypeptides.

EXAMPLE 8

This example illustrates the contemplated use of the 30 kDa protein to generate antibodies. While the example illustrates preparation of a monoclonal antibody, polyclonal antibodies or other monoclonals, developed from epitopic regions of the 30 kDa polypeptide are readily obtainable by similar procedures.

Monoclonal Antibodies to *Bb* 30 kDa Polypeptide

Bb polypeptide is combined with DNA/cellulose and taken up in Freund's complete adjuvant for the initial

immunization. Subsequent immunizations utilize incomplete Freund's adjuvant. BALB/C mice are immunized intraperitoneally initially, then intramuscularly. Blood is checked for testing of antibody production. High antibody titer animals are selected and the spleen removed, minced and cells isolated and tested for viability. Spelenic lymphocytes are then fused with a non-secretor myeloma cell line such as P_3-NS1-Ag4-1 obtained from a commercial source, using PEG to induce cells to fuse. Cells are plated and HAT media used for feeding cultures. Cells are weaned from growth on serum after 2 or more clonings.

Preliminary screening is accomplished by an ELISA. A hybridoma screening kit may be used (*e.g.*, BRL, Bethesda, MD). Plates are coated with goat serum and then hybridoma culture supernatant added to control plates and to plates previously coated with 30 kDa antigenic polypeptide. After incubation, plates are washed and β-galactosidase conjugated goat anti-mouse antibody 1:200 dilution in PBS containing 1% goat serum (BRL reagent) added and further incubated. A chromophoric substance, p-nitrophenyl glucose is added and incubation continues for about 1 hr followed by quenching by addition of sodium carbonate solution. Wells are read at 410 nm on an ELISA plate reader. A positive reaction is indicated by development of a yellow color in the well.

Cells are cloned from positive wells by plating at 0.5-2 cells per well with later recloning at 0.3 to 0.5 cells per well. Positive clones are recognized by a

identification kit. Two antigens are used to coat the plates. Cappel's affinity purified goat anti-mouse IgG-

heavy and light chain at a 1:50 dilution are used. The second antigen is the 30 kDa antigenic *Bb* polypeptide. Once the hybridoma cells are successfully cloned, they may be grown in bulk. Antibody concentrations that might be expected are 10-100 μl/ml.

EXAMPLE 9

This example illustrates a contemplated immunodiagnosis for detection of Lyme disease. This particular example is based on an ELISA type assay, but other types of immunoassays are also contemplated. It will be appreciated that the availability of a protein specific for virulent forms of Lyme disease and monoclonal antibodies to that protein or epitopes having antigenic properties enables development of specific tests for the disease so that immunoassays are not limited to use of the 30 kDa antigen.

ELISA Assay for Lyme Disease

The 30 kDa protein or portions thereof will be produced in large quantities by recombinant DNA vectors and purified. Alternatively, synthetic peptides could be used as antigen. Optimal concentration of the antigen will be determined by checkerboard titration, using serial two-fold dilutions. The antigen in 50 μl of distilled water or 0.05 M $NaHCO_3$ will be added to polystyrene microtiter plates and allowed to dry by incubation for 18-20 hrs at 37°C. Wells incubated with buffer alone will serve an antigen controls. Plates will be washed 3 times with PBS-0.05% Tween 20 prior to use.

Incubation for 1 hr at 37°C in a humidified chamber, the plates will be washed 5 times and incubated with the

optimal dilutions of goat anti-human IgM or anti-human IgG alkaline phosphatase conjugates (1:500 to 1:2000). p-Nitrophenyl phosphate will be used as the substrate, and the reaction stopped at 30 min with 50 µl 3 N NaOH. Absorbance will be measured at 405 nm using a Dynatech ELISA reader. PBS-Tween will be used as the diluent throughout; the possible background-damping effects of using the more complex dilution buffer (Magnarelli *et al.*, 1984) for the blocking step and throughout all the incubation steps will be evaluated. Standard immunofluorescence assay (IFA) and ELISA assays using whole *Bb* will be used for comparison. Depending on the reproducibility of results obtained with repeated ELISAs, reactivity cutoffs will be established as either a certain difference in absorbance (*e.g.*, 0.2) over the negative control wells, or 3 standard deviations above the negative control wells. The titer of a serum will be defined as the reciprocal of the last dilution showing reactivity.

Once optimal conditions are established, a panel of ~100 well-defined sera, potentially including documented true-positive (early and late infections), true negative, false positive and false-negative sera, will be tested for reactivity and compared to the results of the IFA and whole *Bb* ELISA assays. Immunoblot reactivity will also be determined. Specificity and sensitivity of the 30K assay may be examined further by testing sera from mice at different stages of infection and infected with different strains of *Bb*. These results would indicate the relative course for seroconversion for each of the assays and would also show whether infection with

identified by testing peptide fragments generated from isolated 30 kDa protein or, alternatively, by Kyte-

Doolittle analysis of the amino acids of the protein. Hydrophilicity values of ≥ 1.0 were between amino acid segments 22-54, 67-87, 111-115, 128-134, 153-178, 184-186 and 209-226. Using Jameson-Wolf antigenicity and Kyte-Doolittle (1982) analysis to predict antigenic regions indicated regions 23-54, 64-87, 106-114, 128-133, 152-188 and 208-226. Hydrophilicity analysis was used to identify hydrophilic regions of the 30 kDa protein, Hopp, *et al.*, (1981).

EXAMPLE 10

The complete sequence of the DNA encoding the virulence associated 30 kDa protein in *Bb* infections has been determined. Thus primers to this DNA segment are readily developed and PCR methods may be utilized to amplify the 30K DNA or segments of that DNA in biological samples, such as tissue, blood or serum. The following example illustrates a PCR technique contemplated as useful for the sensitive determination of the presence of 30K DNA in samples taken from infected individuals.

Detection of *Bb* in Individuals Suspected of Having Lyme Disease

Oligonucleotide primers are prepared from segments of sense or antisense strands of the DNA sequence. The primers are synthesized by standard phosphoramidite chemistry. Concentrations of Taq DNA polymerase (Perkin Elmer/Cetus, Norwalk, CT), oligonucleotide primers, deoxyribonucleotides (Pharmacia, Piscataway, NJ) and Mg^{2+} as well as amplification cycle lengths, numbers and temperatures are optimized.

Volumes of 100 µl. A typical reaction may contain 50 mM KCL, 10mM Tris-HCl, 3 mM $MgCl_2$, pH 8.3, 100 µ/ml gelatin,

70 picomoles of primers, 300 μM deoxyribonucleotides, 2.5 U of Taq polymerase and a sample of biological fluid suspected of containing *Bb* and which has been treated to assure access of primers to the target DNA (cell lysis, for example). Reactions are performed for 40 cycles in a Thermocycler (Perkin Elmer/Cetus) using the following: (1) denaturation for 1 min 15 sec at 94°C, (2) annealing for 1 min 15 sec at 60°C, and (3) extension for 1 min at 72°C for an additional 10 min and then stored until analysis. To prevent false-positives due to contamination, all specimens are prepared under a laminar flow hood in a PCR dedicated facility remote from the principal laboratory.

Samples are analyzed by removing one tenth of the reaction volume (10 μl) and electrophoresing through a 1% agarose gel containing 0.089 M Tris-HCl, 0.089 M borate, 0.002 M EDTA (TBE) buffer and staining with 0.25 μg/ml ethidium bromide. Gels are photographed under ultraviolet light and, in some cases, transferred to 0.2 μm pore size nitrocellulose for Southern blot analysis. Other specimens may be applied directly to nitrocellulose using a Minifold I apparatus for dot blot DNA-DNA hybridization.

DNA probes for hybridizations are prepared from any of numerous selected DNA segments from the DNA shown in Figure 2. The probe is radiolabeled with [α-^{32}P]dCTP using random hexanucleotide labeling. Southern and dot blot hybridizations are performed overnight at 65°C in 1 M NaCl, 10% dextran sulfate, 1% SDS, 100 μg/ml sheared salmon sperm DNA and with 1 x 10^8 cpm/ml of labeled

x-ray film for one to 24 hrs depending on the specific activity of the probe.

EXAMPLE 11

Computer analysis of photographs of two-dimensional protein gels of *B. burgdorferi* proteins has led to the identification of a 38.8 kDa protein that is present in highly infectious clones of the Sh2 strain of this organism, but is absent from clones that are either non-infectious or have a markedly decreased ability to infect laboratory animals (C3H/HeN mice).

Isolation and Identification of a 38 kDa Protein from Infectious *B. burgdorferi*.

Clonal populations of *B. burgdorferi* Strain Sh2, Passage 5 originated from colonies obtained by subsurface plating of *B. burgdorferi* suspensions in low-melting point agarose. Ten clones were tested for their ability to infect C3H/HeN mice by injecting 10_5 organisms subcutaneously at the base of the tail. Two to four weeks later, the mice were sacrificed and tissue specimens from the tibiotarsal joint, heart, and urinary bladder were cultured in BSKII medium to determine if viable organisms were present. The *B. burgdorferi* clones fell into two classes: high-infectivity and low-infectivity (see Tables 1-5). Sh2 clones 5A3, 5A4, 5A5, 5A6, 5A8, 5A9, and 5A10 were of the high-infectivity phenotype, *i.e.* could be consistently recovered from every organ from the infected mice. Sh2 clones 5A1, 5A2, and 5A3 could only occasionally be recovered from the organs of infected mice, and were thus termed low-infectivity clones. The median infectious doses of the 5A5 and 5A1 clones were determined to be 1.8×10^2

Ta Infectivity screening of clonal populations from *B. burgdorferi* strains Sh2 (passages 5, 10, and 20) and B31 (passage 5).

from mice infected with each clone (10^5/mouse) were scored as either positive (+) ve (-) for growth of *B. burgdorferi*

	Tissue	Culture Results for *B. burgdorgeri* Clones[a] 1	2	3	4	5	6	7	8	9	10	Clones + Total[b]	All Sites[c]
S	Joint	-	-	+	+	+	+	-	+	+	+	7/10	7/10
P 5	Heart	-	-	+	+	+	+	-	+	+	+	7/10	
S	Joint	-	-	-	-	-	+	-	-	-	-	1/10	1/10
P 10	Heart	-	-	-	-	-	+	-	-	-	-	1/10	
S	Joint	-	-	-	-	-	-	-	-	-	-	0/10	0/10
P 20	Heart	-	-	-	-	-	-	-	-	-	-	0/10	
B	Joint	+	-+	+	+	-	+	-	+	+	-	7/10	7/10
B 5	Heart	+	-	+	+	-	+	-	-	+	-	5/10	
	Blood	+	-	+	-	-	+	-	-	+	-	4/10	

a s designated as Sh2 5A1 through 5A10 (passage 5, clones 1 through 10), Sh2 10A1 gh 10A10 (passage 10, clones 1 through 10), Sh2 20A1 through 20A10 (passage 20, s 1 through 10), and B31 5A1 through 5A10 (passage 5, clones 1 through 10)

b = combined number of clones positive for that organ (e.g. joint)

c = number of clones positive at any organ site tested.

Verification of high- and low-infectivity phenotypes of *B. burgdorferi* Sh2

Groups of 5 C3H/HeN mice were inoculated with each clone (10^5/mouse); after 2 weeks the mice were sacrificed and organs cultured for detection of viable *B. burgdorferi*.

Clone	Number of Cultures Positive/Total			Cultures Positive/ Total	Mice Positive/ Total
	Joint	Heart	Bladder		
Infectivity phenotype:					
Sh2-5A3	5/5	5/5	5/5	15/15	5/5
Sh2-5A4	5/5	5/5	5/5	15/15	5/5
Sh2-5A5	5/5	5/5	ND[a]	10/10	5/5
Infectivity phenotype:					
Sh2-5A1	3/5	0/5	ND	3/10	3/5
Sh2-5A2	2/5	0/5	0/5	2/15	2/5
Sh2-5A7	0/5	0/5	0/5	0/15	0/5

[a] Not determined

Infectivity screening of subclones derived from *B. burgdorferi* low-infectivity 2-5A1 and high-infectivity clone Sh2-5A5. Cultures from mice infect with each ue scored as being either positive (+) or negative (-) for growth of *B.* *eri*.

Tissue	Culture Results for Subclones										Clones + Total[b]	All Sites[c]
	1	2	3	4	5	6	7	8	9	10		
Joint	-	-	-	-	-	-	+	-	+	-	2/10	2/10
Heart	-	-	-	-	-	-	-	-	-	-	0/10	
Joint	+	+	-	+	+	+	+	+	+	+	10/10	10/10
Heart	+	+	+	+	+	+	+	+	+	+	10/10	

Infectious Dose-50 (ID_{50}) determination for high infectivity clone Sh2-5A5. The 1.8×10^2 organisms, based on the method of Reed and Munsch.

m	Cultures Positive/Total				
	Joint	Heart	Bladder	All Sites	Mice Positive/Total
	3/3	3/3	3/3	9/9	3/3
	2/3	3/3	3/3	8/9	3/3
	3/3	3/3	3/3	9/9	3/3
	1/3	1/3	1/3	3/9	1/3
	0/3	0/3	0/3	0/9	0/3
Medium	0/3	0/3	0/3	0/9	0/3

Tal
II

Infectious Dose-50 (ID_{50}) determination for low infectivity clone Sh2-5A1. The
0^5 organisms, based on the method of Reed and Munsch.

	Cultures Positive/Total				
	Joint	Heart	Bladder	All Sites	Mice Positive/Total
	0/3	0/3	0/3	0/9	0/3
	2/3	0/3	0/3	2/9	2/3
	1/3	1/3	0/3	2/9	1/3
	0/3	0/3	0/3	0/9	0/3
	0/3	0/3	0/3	0/9	0/3
B	:m 0/3	0/3	0/3	0/9	0/3

The protein content of these clones was analyzed by 2-dimensional gel electrophoresis and silver staining, as described previously (Norris, *et al.*, 1992). The non-equilibrium pH gradient electrophoresis gels used in the first dimension were prepared and the second dimension sodium dodecyl sulfate-polyacrylamide electrophoresis gels were performed. Figures 1A and 1B and Figures 2A-2F show a portion of the gel pattern containing the majority of the acid, neutral, and basic proteins. The high-infectivity clone Sh2 5A5 (Fig. 1A) was found to contain a moderately abundant, 38.8 kDa protein spot that migrated underneath the 41 kDa flagellin protein, whereas the low-infectivity clone Sh2 5A1 (Fig. 1B) either lacked or expressed greatly reduced quantities of this protein. A more detailed analysis indicated that the high-infectivity clones 5A5, 5A3, and 5A4 all expressed a protein with the same relative molecular mass (Mr) and isoelectric point as the 38.8 kDa protein (Figs. 2A-C), and the low infectivity clones 5A1, 5A2, 5A7 had little or no detectable protein in this gel location (Figs. 2D-F). Other moderately abundant proteins just below the flagellin protein and below the 38.8 kDa protein were present consistently in all gel patterns.

Further characterization will include isolation of the protein by preparative 2-dimensional gel electrophoresis and use of this material to produce specific antiserum and to obtain amino acid sequence data. The gene will then be isolated either by screening a *B. burgdorferi* recombinant DNA library for clones expressing a protein reactive with the specific antiserum, screening the library using [illegible] sequence data, using well known techniques. Once isolated, large quantities of the protein will be

expressed in *E. coli* using expression vectors, in a manner analogous to that employed for the 30 kDa protein.

Uses of the new 38.8 kDa polypeptide include all those described for the 30 kDa protein, *e.g.* detection of specific antibodies in serum of patients or animals with Lyme borreliosis, use of all or part of the protein as an immunogen to protect against infection, and use of related nucleotide sequences or specific antibodies for detection of Lyme borreliae in mammal or arthropod tissue, body fluids, or cultured extracts thereof.

****

The present invention has been described in terms of particular embodiments found by the inventors to comprise preferred modes of practice of the invention. It will be appreciated by those of skill in the art that in light of the present disclosure numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. All such modifications are intended to be included within the scope of the claims.

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The references listed below are incorporated herein by reference to the extent that they supplement, explain, provide a background for or teach methodology, techniques and/or compositions employed herein.

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CLAIMS

1. A polypeptide having a molecular weight of about 38 kDa as determined by two-dimensional gel electrophoresis and which is produced in infectious *B. burgdorferi*.

2. The polypeptide of claim 1 wherein the infectious *B. burgdorferi* are found in tissue and body organs following administration of about 10_5 organisms.

3. The polypeptide of claim 1 wherein the *B. burgdorferi* is strain Sh2.

4. The polypeptide of claim 2 wherein the Sh2 is 5A3, 5A4 or 5A5.

5. A method of detecting the presence of Lyme disease comprising determining the presence of antibodies to the polypeptide of claim 1 in the blood of subjects suspected of harboring Lyme disease.

FIGURE 1A

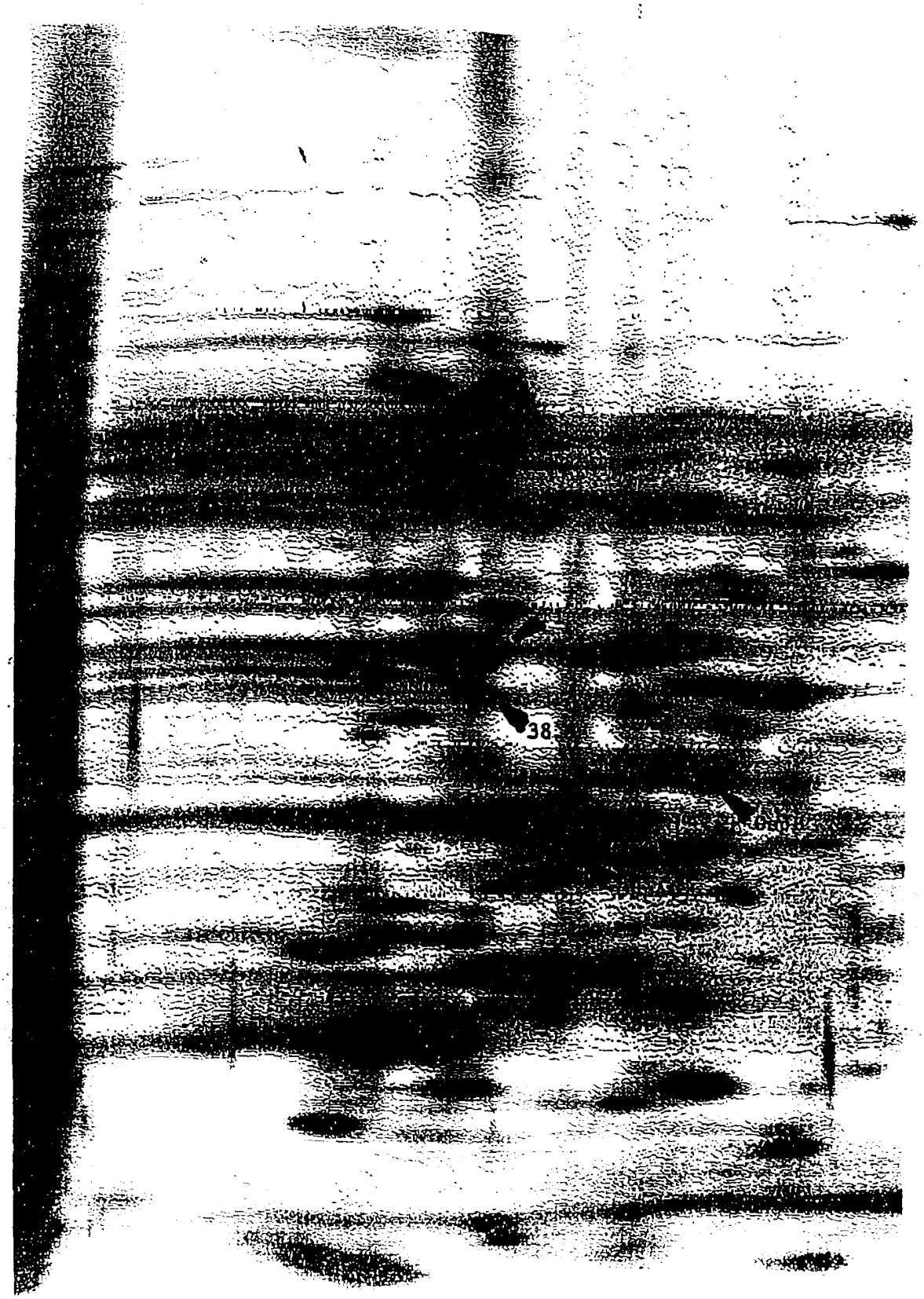

FIGURE 1B

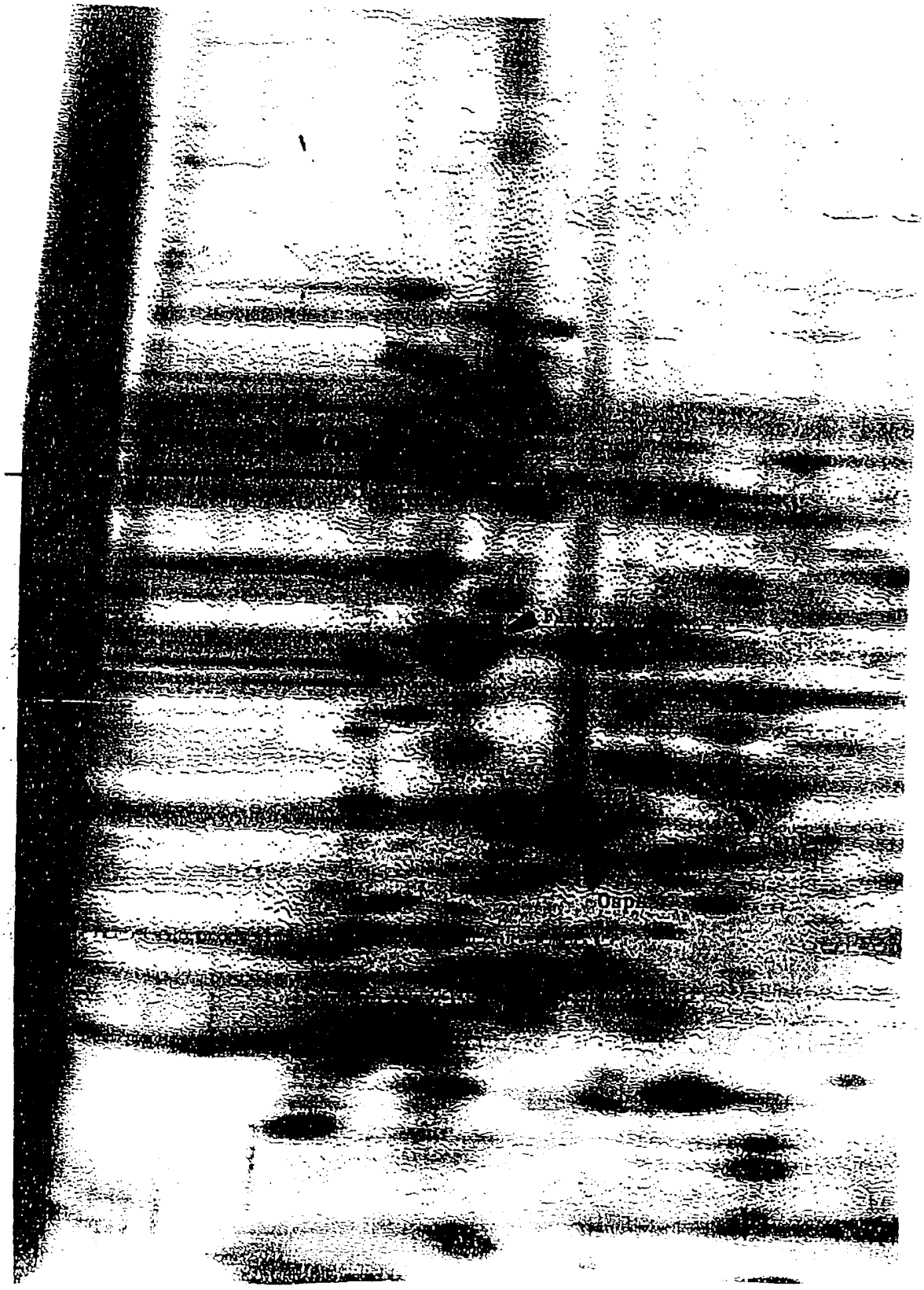

FIGURE 2A

FIGURE 2B

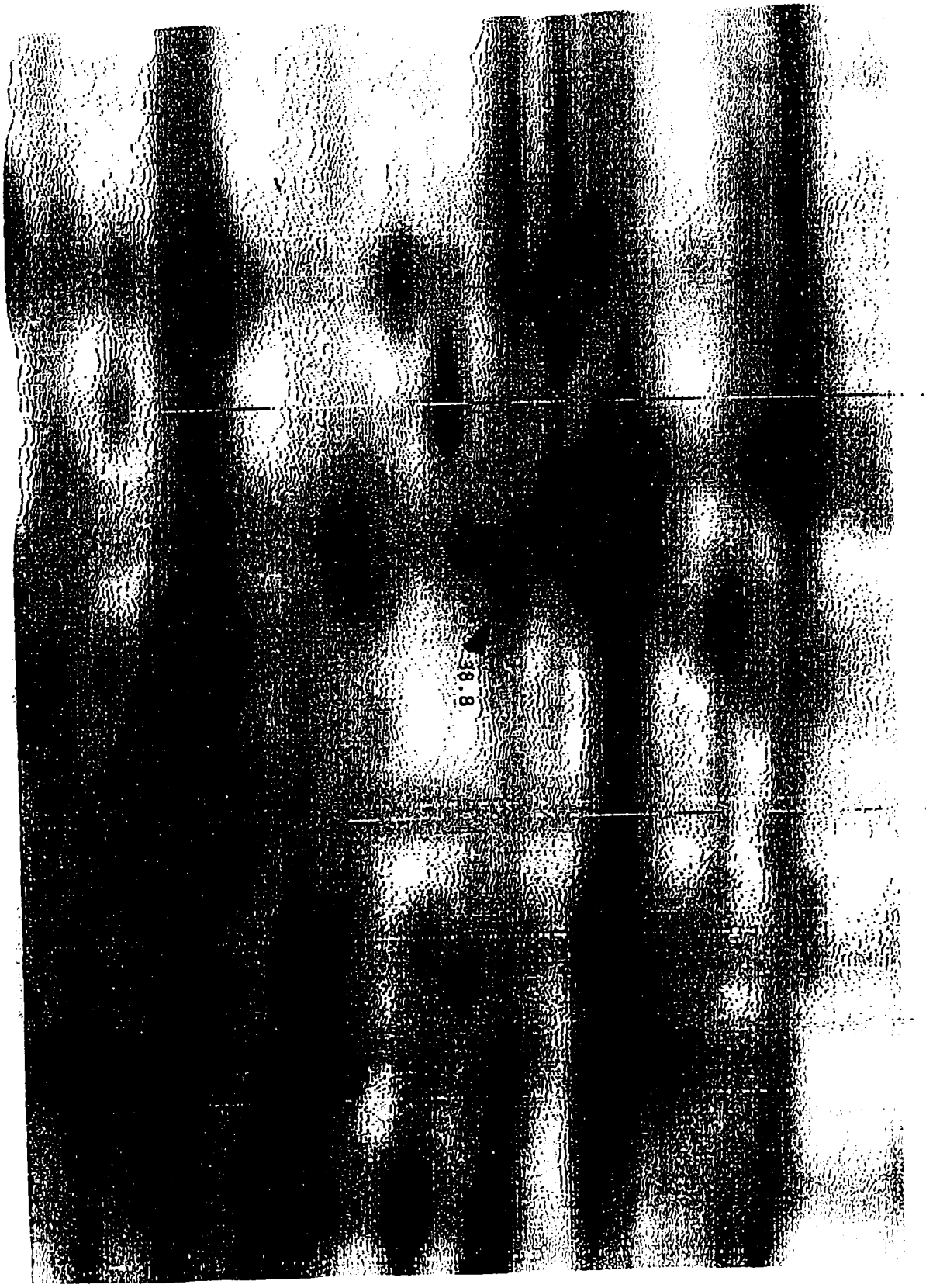

FIGURE 2C

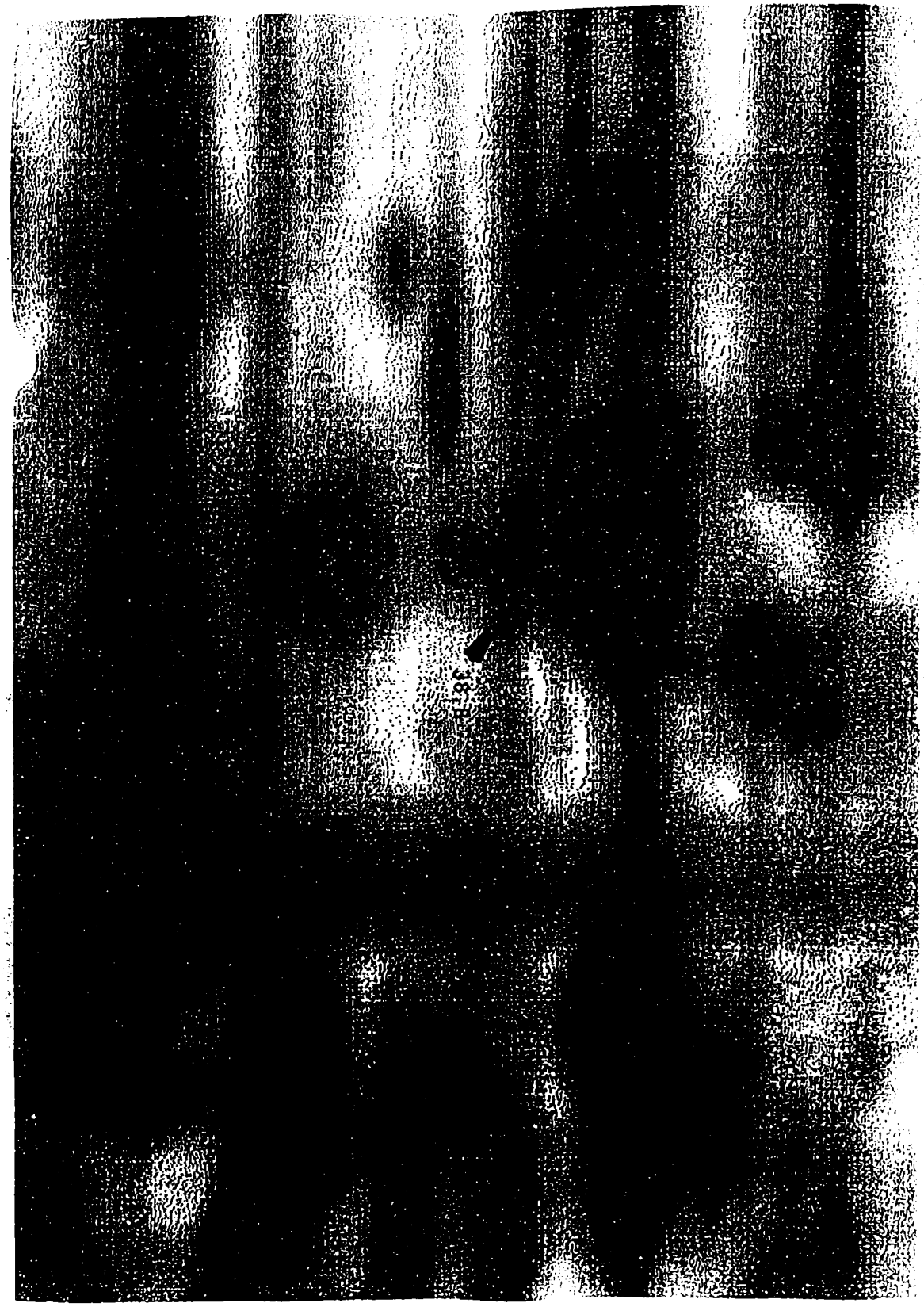

FIGURE 2D

FIGURE 2E

FIGURE 2F

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/10729

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 14/20; G01N 33/569
US CL :530/403, 350, 825; 436/518
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/403, 350, 825; 436/518

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline, Biosis, Embase, Derwent

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A, 91/13630 (SIMPSON ET AL.) 19 September 1991, see pages 18-19.	1-5
X	Journal of Clinical Microbiology, Volume 29, No. 2, issued February 1991, Simpson et al, "Antibody to a 39-kilodalton *Borrelia burgdorferi* Antigen (P39) as a Marker for Infection in Experimentally and Naturally Inoculated Animals", pages 236-243, see pages 236-237 and 240.	1-5
X,P	Infection and Immunity, Volume 61, No. 10, Issued October 1993, Scriba et al, "The 39-kilodalton Protein of *Borrelia burgdorferi*: a Target for Bactericidal Human Monoclonal Antibodies", pages 4523-4526, see pages 4523-4526.	1, 5

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
01 NOVEMBER 1994	12 JAN 1995

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/10729

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Journal of Rheumatology, Volume 20, No. 4, issued 1993, Fawcett et al, "Detection of Antibodies to the Recombinant P39 protein of *Borrelia burgdorferi* using Enzyme Immunoassay and Immunoblotting", pages 734-738, see pages 736-737.	1, 5